

● PERSPECTIVE

HSP70 cleavage-induced photoreceptor cell death caused by *N*-methyl-*N*-nitrosourea

Retinal degenerative diseases (RDs) such as retinitis pigmentosa (RP) are characterized by slowly progressive photoreceptor cell death, but the molecular mechanism underlying RP remains unclear. Animal models for RP have led to a better understanding of the disease pathological mechanisms, yet it remains difficult to identify an appropriate genetic model for RDs in general because there are many causative genes (Rossmiller et al., 2012).

N-methyl-*N*-nitrosourea (MNU), a direct-acting alkylating agent, induces selective photoreceptor cell loss and significantly decreases the outer nuclear layer (ONL) thickness within one week of intraperitoneal injection. This model has been investigated extensively in rodents. Herrold et al. (1967) originally reported that MNU causes photoreceptor cell loss in golden hamsters. Injection of 60 mg/kg MNU into rats or mice also evoked photoreceptor cell loss and dramatically decreased ONL thickness. Tsubura's group found that TUNEL-positive cell death peaked at 3 days after MNU treatment and essentially complete photoreceptor cell loss within one week of MNU treatment (Tsubura et al., 2010). Many reports have suggested that MNU induces the generation of free radicals and cell death specifically in photoreceptor cells.

Oxidative stress is involved in the pathogenesis of a number of neurodegenerative disorders such as RP (Tsuruma et al., 2011; Furukawa and Koriyama, 2016). In particular, the eye has 3- to 4-fold higher oxygen consumption compared to brain tissue and, consequently, has a higher exposure to reactive oxygen species (ROS) such as hydrogen peroxide, hydroxyl radicals, and super oxide anions. Free radicals are highly reactive species which, once formed, can start a series of reactions that are harmful to neural cells. A main target of free radicals is the membrane lipid bilayer, and particularly the polyunsaturated fatty acid (PUFA) chains in lipids. Oxidation of PUFAs in cellular membranes *via* free radical chain reactions generates lipid hydroperoxides as the primary products, and some of these primary oxidation products may decompose and lead to the generation of reactive lipid electrophiles. 4-Hydroxy-2-nonenal (4-HNE) is a reactive aldehyde species generated endogenously from decomposition of the hydroperoxide of ω -6 PUFAs. 4-HNE is highly reactive and may be considered as a secondary toxic messenger that disseminates and potentiates initial free-radical events. Upon reaction with a protein, 4-HNE specially reacts with nucleophilic amino acids such as cysteine, histidine and lysine to form their Michael addition adducts possessing a carbonyl functionality. Protein carbonylation can affect the function and/or metabolic stability of various proteins. Carbonylated proteins are removed by proteolytic degradation or accumulate as damaged or unfolded proteins because carbonylated proteins cannot be repaired. Tsuruma et al. (2012) reported that MNU induces the production of free radicals, the accumulation of 4-HNE, and cell death in photoreceptor cells. In addition, MNU causes a decrease in reduced glutathione, which effectively scavenges free radicals and other ROS, leading to an imbalance between the production of ROS and antioxidants. Thus, oxidative stress plays a pivotal role in MNU-induced retinal photoreceptor degeneration. 2,4-Dinitrophenyl hydrazine (DNPH) reacts with protein carbonyls to form protein-bound 2,4-dinitrophenyl hydrazone (DNP) (Nakamura and Goto, 1995). Protein carbonylation can be detected by using anti-DNP antibody (Figure 1a and b). The accumulation of 4-HNE was observed 1–2 days after treating mouse retina with MNU (Koriyama

et al., 2014) and then protein carbonylation was detected in photoreceptor cells 3 days after treatment. Thus, the accumulation of 4-HNE plays a key role in the neurodegenerative mechanism of MNU-induced photoreceptor cell death.

Calcium ion is an important ion for cellular signaling. Once calcium ions enter the cytoplasm, they can activate the activities of many proteins such as calcium ion-dependent enzymes. Calpain is ubiquitously expressed in the central nervous system and is a calcium ion-dependent protease involved in signaling pathways that modulates the activity and/or function of the substrate proteins. There are two conventional calpain species in mammals: μ -calpain and m -calpain. These calpains differ in calcium sensitivity *in vitro* and are activated at micromolar and millimolar concentrations of calcium, respectively. Under pathological conditions, such as in the animal model of RP, rd10 (Rossmiller et al., 2012) which has a mutation in the rod photoreceptor-specific cGMP phosphodiesterase, intracellular calcium ion levels increase in photoreceptor cells prior to the detection of apoptotic cells. Increased photoreceptor cell death in rd10 mice, another mutation model of RP retina, is associated with calcium ion overload and calpain activation, both of which are seen prior to signs of cell loss. μ -Calpain and cathepsin D contribute to the activation of Bax and apoptosis-inducing factor in various RP model mice. After MNU treatment, total calcium ion was increased in the retina (Oka et al., 2007). Furthermore, MNU-induced photoreceptor cell death was protected by inhibiting calpain activation (Koriyama et al., 2014). Thus, calcium ion-dependent calpain activation may play an important role in the MNU-induced photoreceptor cell death model. MNU-induced photoreceptor cell loss is caused by activation of caspase cascades after MNU injection (Tsuruma et al., 2012). Although such molecular mechanisms of MNU-induced photoreceptor cell loss have been described, the total process of photoreceptor cell death signaling remains unknown. Thus, elucidation of the key molecule that connects these cell death molecular mechanisms is necessary to reveal the cell death signaling process caused by MNU.

The heat shock protein 70 (HSP70) family is conserved and ubiquitously expressed. HSP70 is a central component of the cellular network of molecular chaperones and folding catalysis (proteostasis) regulators and protects cells from various stresses (Furukawa and Koriyama, 2016). In the normal rat eye, HSP70 immunoreactivity is localized in the outer nuclear layer and the inner segments of the retina. HSP70 prevents photoreceptor apoptosis after retinal detachment through prolonged activation of Akt, a survival signaling pathway (Kayama et al., 2011). On the other hand, under pathological conditions of neuronal tissues, such as glaucoma and ischemia/reperfusion of hippocampus, HSP70 is a known common substrate of calpain. HSP70 carbonylated by 4-HNE is much more vulnerable to calpain cleavage. For example, calpain-mediated cleavage of HSP70 results in lysosomal membrane rupture and cell death through cathepsin-dependent proteostasis disruption because HSP70 stabilizes lysosomal membranes (Yamashima, 2012). We showed that levels of 4-HNE clearly increased in MNU-injected mouse retina. Moreover, HSP70 was rapidly and calpain-dependently cleaved after MNU treatment (Koriyama et al., 2014). HSP70 cleavage subsequently led to photoreceptor cell death (Figure 1c). In addition, we reported that well known HSP70 inducers such as valproic acid attenuated photoreceptor cell death caused by MNU (Furukawa and Koriyama, 2016). Co-administration of HSP70 inducer and HSP inhibitor abolished the protective effect of HSP70, indicating that HSP70 plays a crucial role in protecting cells from death due to MNU. Calpain inhibitor also protects photoreceptor cells by preventing HSP70 cleavage. In addition, we recently reported that geranylgeranylacetone, another HSP70 inducer, also attenuates photoreceptor cell death

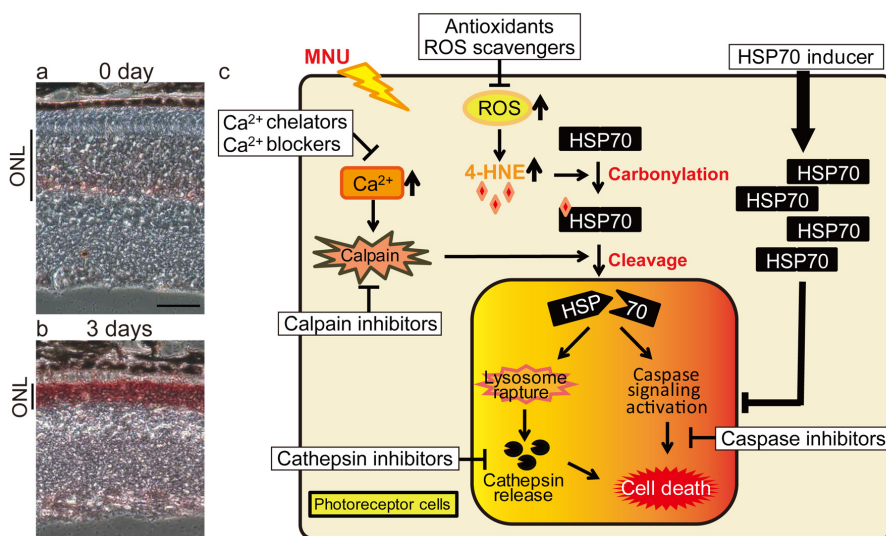


Figure 1 The mechanism of *N*-methyl-*N*-nitrosourea (MNU)-induced photoreceptor cell death and of candidate therapeutic agents for retinitis pigmentosa (RP). (a) Protein carbonylation levels in vehicle control retina. Scale = 50 μ m. (b) Protein carbonylation levels in retina treated for 3 days with MNU. (c) We propose that heat shock protein 70 (HSP70) is rapidly and calpain-dependently cleaved after MNU treatment. In addition, we previously reported that HSP70 inducers attenuated photoreceptor cell death caused by MNU. ONL: Outer nuclear layer; ROS: reactive oxygen species.

caused by MNU through HSP70 induction (Furukawa and Koriyama, 2016). Thus, HSP70 plays a crucial role in the maintenance of photoreceptor cells, and HSP70 inducers may be powerful therapeutic agents. Based on the overall mechanism of photoreceptor cell death proposed by us, protein carbonylation by oxidative stress, calcium ion-dependent protease activation, apoptosis-related molecules, and HSP70 cleavage are involved in MNU-induced photoreceptor cell death. The final common end stage is photoreceptor cell death in RP, and thus therapies to suppress photoreceptor cell death in RP models have been investigated. Antioxidants and ROS scavengers can reduce MNU-induced photoreceptor cell death. For example, *N*-acetylcysteine reduced photoreceptor cell death by reducing oxidative damage in RP models (Furukawa and Koriyama, 2016). Even in MNU-injected mice retina, edaravone treatment can reduce 4-HNE accumulation and the number of TUNEL-positive cells (Tsuruma et al., 2012). Furthermore, polyphenols such as curcumin and green tea extract, and nimodipine, a calcium blocker, reduced the number of MNU-induced TUNEL-positive photoreceptor cell killed (Furukawa and Koriyama, 2016). Moreover, two inhibitors of calpain (ALLN, SNJ-1945) and cathepsin (CA-074Me, E-64) can attenuate photoreceptor cell loss. The final common end stage of various pathogenic mechanisms in retinal degenerative disease is the apoptotic cell death of photoreceptor cells. Therefore, caspase inhibitors are also thought to be effective therapeutic tools for photoreceptor cell death. A caspase inhibitor suppressed retinal apoptosis in MNU-treated rats. In addition to these existing therapies for RP, we were the first to propose that HSP70 inducers can be therapeutic agents for suppressing photoreceptor cell death in RP. Many chemicals have been reported as HSP70 inducers, including alkannin, arimoclomol, celastrol and curcumin (Furukawa and Koriyama, 2016). In our recent studies, we proposed that HSP70 carbonylation by 4-HNE and the subsequent cleavage of HSP70 by calpain activation plays a role in photoreceptor cell death (Yamashima, 2012). In addition, HSP70 inducers protected against photoreceptor cell death caused by MNU by maintaining proteostasis (Koriyama et al., 2014). Taken together, HSP70 inducers may be candidate therapeutic agents for the prevention of photoreceptor cell death caused by MNU.

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